FORM PTO-1390 (REV 11-2000)	U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 159-68
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/914292 Unknown
INTERNATIONAL APPLICATION NO. PCT/JP99/01392	INTERNATIONAL FILING DATE 19 March 1999	PRIORITY DATE CLAIMED

TITLE OF INVENTION

METHOD OF QUANTIFYING SUBSTRATE AND BIOSENSOR

APPLICANT(S) FOR DO/EO/US

SHINOZUKA et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The U.S. has been elected by the expiration of 19 months from the priority date (Article 31).
5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   - b. ☒ has been communicated by the International Bureau.
   - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the PCT Request and the International Application as filed (35 U.S.C. 371(c)(2)).
   - a. ☒ is attached hereto.
   - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   - b. ☐ have been communicated by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has **NOT** expired.
   - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 To 20 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information. Front page of the PCT Publication

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/914292 Unknown	PCT/JP99/01392	159-68

21. ☒ The following fees are submitted:

	CALCULATIONS	PTO USE ONLY

BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5):

-- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1000.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....$860.00

-- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO.....$710.00

-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4).....$690.00

-- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).....$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =						$	860.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).						$	0.00	
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE				
Total Claims	5	-20 =	0	X	$18.00	$	0.00	
Independent Claims	1	-3 =	0	X	$80.00		0.00	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)					$270.00	$	0.00	
TOTAL OF ABOVE CALCULATIONS =						$	**860.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.							0.00	
SUBTOTAL =						$	**860.00**	
Processing fee of $130.00, for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)). +							0.00	
TOTAL NATIONAL FEE =						$	**860.00**	
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property +						$	40.00	
Fee for Petition to Revive Unintentionally Abandoned Application ($1240.00 – Small Entity = $620.00)						$	0.00	
TOTAL FEES ENCLOSED =						$	**900.00**	
						Amount to be: refunded	$	
						Charged	$	

a. ☒ A check in the amount of $900.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of $_____ to cover the above fees. A duplicate copy of this form is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.
d. ☒ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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25,327 REGISTRATION NUMBER

August 27, 2001 Date

554157

6/parts

SPECIFICATION

METHOD OF QUANTIFYING SUBSTRATE AND BIOSENSOR

FIELD OF THE INVENTION

This invention relates to a method of conveniently and quickly quantifying substrates contained in various samples, for example, biological samples such as blood, urine, saliva and sweat, foods and environmental samples and a biosensor. More particularly, it relates to a method of quantifying a substrate through reactions by using an electrode system made of electrically conductive materials and various reagents and a biosensor with the use of the same.

BACKGROUND OF THE INVENTION

It has been considered that methods of quantifying substrates by using dehydrogenases and coenzymes are useful in the field of analytical chemistry for clinical examinations, food analysis, etc. An enzyme reaction with the use of a dehydrogenase and a coenzyme as catalysts means a reaction whereby a substrate contained in a sample is specifically oxidized and, at the same time, the coenzyme is reduced. There have been confirmed several hundred dehydrogenase reactions occurring *in vivo*. These enzyme reactions are highly important because they are applicable to the quantification of substrates in samples, the measurement of enzyme activities, etc. In these measurement methods, reduced coenzymes formed by the

reactions are detected.

These reduced coenzymes formed as the reaction products are quantified by liquid chromatography (Analytical Biochemistry, Vol.146, p.118 (1985)), UV absorption spectroscopy (Clinical Chemistry, Vol.22, p.151 (1976)) and the like. Use is also made of a method which comprises subjecting a reduced coenzyme to a redox reaction with an oxidant selected from among tetrazolium salts (Japanese Patent Public Disclosure No.286784/97, Analyst, Vol. 120, p.113(1995)), ferricyanides, quinones, cytochromes, metal ions, etc. and then quantifying the reduced product thus formed by the absorption spectroscopy in the visible region. However, none of these methods enables convenient and quick measurement, since it is needed therein to perform pretreatments such as dilution or separation. Another problem is that large-scale and expensive measurement apparatuses are needed when employing these methods.

In recent years, there have been employed biosensors of electrochemical detection type as means of conveniently and quickly quantifying reduced coenzymes formed by enzyme reactions. In these cases, it is anticipated that reduced coenzymes would be directly detected electrochemically (Analyica Chimica Acta, Vol.336, p.57 (1996)). However, reduced coenzymes can hardly undergo redox reactions via electron transfer. Therefore, it is necessary to apply a high potential to directly oxidize a reduced coenzyme on electrodes. However, the application of such a high

potential causes pollution and damage of the electrodes or induces effects of coexisting matters. Attempts to solve these problems have been made by using electron mediators as can be seen from a number of reports and patents concerning biosensors published so far (Japanese Patent Public Disclosure No.165199/98). Examples of electron mediators employed in biosensors at present include phenazine derivatives such as 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS) (Analyst, Vol.119, p.253 (1994)), Meldola's Blue (Analytica Chimica Acta, Vol.329, p.215 (1996)), ferricyanides (Analytical Chemistry, Vol.59, p.2111 (1987)), ferrocene (Analytical Chemistry, Vol.70, p.4320 (1998)) and quinones (Bioscience & Bioelectronics, Vol.11, p.1267 (1996)). Such an electron mediator is reduced by a redox reaction with a reduced enzyme and the reduced electron mediator thus formed easily undergoes a redox reaction by applying a potential on electrodes. Therefore, detection can be made by applying a lower potential, compared with the case of oxidizing a reduced coenzyme directly on electrodes.

The present inventors have devised biosensors of an integrated type consisting of a reaction reagent, which comprises various dehydrogenases, oxidized nicotinamide adenine dinucleotide (NAD^+) as a coenzyme and an electron mediator 1-methoxy PMS, with an electrode system (Japanese Patent Application No.201553/98; PCT/JP98/03194) and constructed biosensors whereby various substrates can be conveniently and quickly quantified. In these biosensors,

an absorbent carrier carrying all of the reaction reagents is located between a working electrode and a counter electrode which are made of electrically conductive materials and formed by the printing method. It is confirmed that a highly favorable linear response current depending on the concentration of each substrate can be obtained thereby. However, subsequent studies have revealed that these biosensors still suffer from the problem. Namely, the response current in the low substrate concentration region is liable to be affected by coexisting matters. This is seemingly because electron mediators are chemically unstable due to the very low standard redox potential thereof and, therefore, liable to undergo redox reactions with redox matters coexisting in samples, which results in fluctuation and decrease in the response current in the low substrate concentration region. To conduct highly accurate quantification with a stable response current, it is therefore necessary to further improve the system.

SUMMARY OF THE INVENTION:

To solve the above-described problems, the present invention provides a method of quantifying a substrate by using an electrode system made of electrically conductive materials and a reaction reagent comprising at least a dehydrogenase, a coenzyme, an electron mediator and a tetrazolium salt and a biosensor.

Compared with the conventional methods with the

direct oxidization of reduced coenzymes or the use of various electron mediators, the method according to the present invention makes it possible to reduce the fluctuation in the response current since a chemically stable formazan is formed as the final product. In the method of the present invention, moreover, the response current is largely increased and the detection sensitivity is elevated, which makes it possible to quantify a substrate in the lower concentration region. Consequently, a substrate in a sample can be quantified with high accuracy.

DESCRIPTION OF THE INVENTION

The present invention provides a method of quantifying a substrate by using an electrode system consisting of at least a working electrode and a counter electrode made of electrically conductive materials and a reaction reagent comprising at least a dehydrogenase, a coenzyme, an electron mediator and a tetrazolium salt, and a biosensor in which the reaction reagent and the electrode system are integrated and which enables convenient and quick quantification.

In the present invention, the substrate in the sample undergoes a specific enzyme reaction under the action of the dehydrogenase and the coenzyme contained in the reaction reagent to form a reduced coenzyme. Then a redox reaction quickly proceeds between this reduced coenzyme and the electron mediator and the tetrazolium salt, and a

chemically stable formazan is formed as the final product. Next, the formazan is electrochemically changed by applying a potential to the electrode system and the thus arising response current is detected. Since this response current depends on the substrate concentration, the substrate can be thus quantified. Fig. 5 roughly shows the process of a series of reactions as described above. Fig. 6 shows the fundamental structural formulae of the tetrazolium salt reacting finally and the formazan formed as the final product.

The substrate which can be quantified in the present invention involves any substrates in dehydrogenation reactions whereby reduced coenzymes are formed by using dehydrogenases as a catalyst. Use of such an enzyme reaction makes it possible not only to quantify a substrate but also to measure enzyme activity, etc. Namely, substrates over an extremely large range are usable in the method according to the present invention, which makes it applicable to various measurements. Particular examples of the substrate include alcohols, galactose, glucose, cholesterol, lactic acid, phenylalanine and leucine. However, it is obvious that other various substrates can be quantified by the method of the present invention.

Since a chemically stable formazan is formed as the final product in the method of the present invention, a reduction in the fluctuation response current can be obtained. It has been already confirmed by the above-

described spectroscopy method that the reaction of forming a formazan from a substrate smoothly and quantitatively proceeds (Japanese Patent Public Disclosure No.286784/97, Analyst, Vol.120, p.113 (1995)). According to the present invention, it has been further clarified that detection can be carried out by using an electrode system and thus a more useful quantification method has been established. As a result, a current density of about 120 $\mu A/cm^2$ is established by the biosensor of the present invention and thus the response current is largely increased and the detection sensitivity is improved, since the current density of the conventional biosensors constructed ranges from about 4 to 12 $\mu A/cm^2$ per mM of a substrate and the current densities of the existing biosensors with the use, as the electron mediator, of ferricyanides (Analytical Chemistry, Vol. 59, p.2111 (1987), ferrocene (Analytical Chemistry, Vol.70, p.4320 (1998)) and quinones (Bioscience & Bioelectronics, Vol.11, p.1267 (1996)) are respectively about 2 $\mu A/cm^2$ (calculated from Fig. 6, p.2114), about 6 $\mu A/cm^2$ (calculated from Fig. 4, p.4323) and about 10 $\mu A/cm^2$ (Fig. 10, p.1273). Moreover, the present invention enables quantification of a substrate in a lower concentration region. Thus, a substrate can be quantified at a high accuracy by using the quantification method and biosensor according to the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagram schematically showing the

constitution of the biosensor in an example of the present invention.

Fig. 2 is a graph showing the fundamental responses of the biosensor in Example 2.

Fig. 3 is a graph showing the result of the response to reduced nicotinamide adenine dinucleotide (NADH) in Example 3.

Fig. 4 is a graph showing the results of the response to L-phenylalanine in Example 4.

Fig. 5 is a reaction model view of the present invention.

Fig. 6 shows the structural formulae of tetrazolium salts and formazans.

The symbols given in the above figures have the following meanings: 1 stands for an insulating support; 2 stands for a working electrode; 3 stands for a counter electrode; 4 stands for an insulating layer; and 5 stands for an absorbent carrier.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The electrode system to be used in the present invention may be an arbitrary one without restriction, so long as it is made of electrically conductive materials and is electrochemically stable. Examples of materials usable therefor include carbon, gold, silver, silver/silver chloride (Ag/AgCl), nickel, platinum, platinum black, palladium and alloys of these metals. As the results of examinations on various materials, it has been found out

that carbon materials are favorable as the working electrode in the electrode system of the present invention, since they are less expensive and chemically stable.

The term "carbon materials" as used herein means materials containing carbon. Any carbon materials employed in the conventional carbon electrodes are usable herein without any particular restriction. For example, use can be made of carbon fiber, carbon black, carbon paste, glassy carbon, graphite and the like.

By using such a carbon material, an electrode is formed on the insulating support by a method commonly employed. Usually, the carbon material is made into a paste by using a resin binder, etc., screen-printed and then dried by heating to thereby form the electrode.

The insulating support may be made of glass, glass epoxy, ceramics, plastics, etc., though the material thereof is not restricted thereto so long as it is not damaged in the step of forming the electrodes by printing or adding a sample. For example, it is possible to use plastic films made of polyester, polyethylene, polyethylene terephthalate, poylystyrene, polypropylene, etc. It is found out that polyester films are favorable herein, since they are less expensive and excellent in adhesiveness to conductive inks and processing properties.

The printing method is not restricted to the screen-printing but use may be made of, for example, gravure printing, offset printing or ink jet printing.

The substrate which can be quantified by the method

of the present invention is not particularly restricted, so long as it can form a reduced coenzyme with the use of a dehydrogenase as a catalyst. Namely, any substrate can be quantified. For example, use can be made of alanine, alcohols, aldehydes, isocitric acid, uridine-5'-diphospho-glucose, galactose, formic acid, glycerylaldehyde-3-phosphate, glycerol, glycerol-3-phosphate, glucose, glucose-6-phosphate, glutamic acid, cholesterol, sarcosine, sorbitol, carbonic acid, lactic acid, 3-hydroxybutyric acid, pyruvic acid, phenylalanine, fructose, 6-phosphogluconic acid, formaldehyde, mannitol, malic acid, leucine, etc.

The dehydrogenase to be used in the present invention is not particularly restricted, so long as it is an enzyme capable of forming a reduced coenzyme. The origin of the dehydrogenase is not restricted either. For example, use can be made of alanine dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, isocitrate dehydrogenase, uridine-5'-diphospho-glucose dehydrogenase, galactose dehydrogenase, formate dehydrogenase, glycerylaldehyde-3-phosphate dehydrogenase, glycerol dehydrogenase, glycerol-3-phosphate dehydrogenase, glucose dehydrogenase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, cholesterol dehydrogenase, sarcosine dehydrogenase, sorbitol dehydrogenase, carbonate dehydrogenase, lactate dehydrogenase, 3-hydroxybutyrate dehydrogenase, pyruvate dehydrogenase, phenylalanine dehydrogenase, fructose dehydrogenase, 6-phosphogluconate dehydrogenase, formaldehyde dehydrogenase, mannitol dehydrogenase, malate

dehydrogenase, leucine dehydrogenase, etc.

The electron mediator is not particularly restricted, so long as it can quickly undergo a redox reaction with a reduced coenzyme and a tetrazolium salt. For example, use can be made of quinones, diaphorase, cytochromes, biologen, phenazines, phenoxazines, phenothiazines, ferricyanides, ferredoxins, ferrocene and derivatives thereof, etc. Among all, phenazines show a high response stability. In particular, it has been found out that 1-methoxy PMS is preferable as the electron mediator in the present invention because of its improved storage stability and reactivity with reduced coenzymes and tetrazolium salts.

The tetrazolium salt is not particularly restricted, so long as it can form formazan. Among all, it has been found out that 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) is preferable as the tetrazolium salt to be used in the present invention, since it provides a water-soluble and chemically stable formazan by reduction and the thus formed formazan shows a specific response in the electrode system.

Examples

Now, the invention will be illustrated in greater detail by reference to the following examples. However, it is to be understood that the invention is not construed as being restricted thereto.

Example 1: Construction of biosensor

Fig. 1 is a diagram schematically showing the constitution of the biosensor in an example of the present invention.

On an insulating support 1 made of a polyester film (manufactured by Diafoil Hoechst Co.), a working electrode 2 and a counter electrode 3 were formed by screen-printing respectively using a conductive graphite ink (manufactured by Acheson Japan Ltd.) and a conductive Ag/AgCl ink (manufactured by Acheson Japan Ltd.) followed by drying by heating (60°C, 1 hour), thereby forming an electrode system.

A buffering component, which was employed for regulating the pH value of the enzyme reaction to the optimum level, was adsorbed on the working electrode 2 and fixed by drying (40°C, 15 minutes).

1-Methoxy PMS (manufactured by Dojindo Laboratories Co., Ltd.) serving as the electron mediator was adsorbed on the counter electrode 3 and fixed by drying (40°C, 15 minutes).

WST-1 (manufactured by Dojindo Laboratories Co., Ltd.) employed as the tetrazolium salt, a dehydrogenase and a coenzyme were dissolved in a phosphate buffer (pH 8.0, 20 mM), then adsorbed on an absorbent carrier 5 made of cellulose fiber (manufactured by Advantec Toyo) and fixed by drying (40°C, 15 minutes).

The working electrode 2 having the buffer component fixed thereto and the counter electrode 3 having 1-methoxy PMS fixed thereto were faced to each other and the absorbent carrier containing WST-1, the dehydrogenase and

the coenzyme was located between these electrodes of the electrode system, thereby forming a biosensor.

Example 2: Measurement of the fundamental response of biosensor

Fig. 2 shows the results of the measurement of the fundamental responses of the biosensor constructed in Example 1.

In this example, 5 μL portions of a standard solution containing NADH and another standard solution free from NADH were added to the above-described sensor. Then a formazan was formed by the redox reaction between the added NADH and 1-methoxy PMS and WST-1. The obtained results show the cyclic voltammogram of the formazan (sweep speed: 50 mV/sec; Model HZ-3000 manufactured by Hokuto Denko Corporation). The solid line shows the result obtained by using the standard solution containing NADH (1.5 mM) while the broken line shows the result obtained by using the NADH-free standard solution.

As these results show, an oxidation peak appeared at around +500 mV vs. Ag/AgCl and thus a response current characteristic to formazan could be obtained.

Example 3: Quantification of NADH

Fig. 3 shows the result of the measurement of NADH, which is a reduced coenzyme formed by reacting a sample with a dehydrogenase and a coenzyme, by using the biosensor constructed in Example 1.

Sixty seconds after adding 5 μL of a sample containing NADH, a potential was applied at +700 mV vs. Ag/AgCl (Model HZ-3000 manufactured by Hokuto Denko Corporation) by using the counter electrode as the standard and the response current was measured (Model HZ-3000 manufactured by Hokuto Denko Corporation).

As a result, a response of a very good linearity was achieved in an NADH concentration range of from 0 to 1.5 mM.

Thus, it is expected that the quantification method and biosensor according to the present invention are applicable to enzyme reactions with the use of any dehydrogenases and coenzymes forming reduced coenzymes.

Example 4: Quantification of L-phenylalanine

Fig. 4 shows the result of the measurement of a standard solution containing L-phenylalanine with the use of the biosensor constructed in Example 1 by reference to Example 3.

In this example, 5 μL of a standard solution containing L-phenylalanine was added to a biosensor constructed with the use of L-phenylalanine dehydrogenase (EC 1.4.1.20, manufactured by Unitika Ltd.). After 60 seconds, a potential was applied at +700 mV vs. Ag/AgCl by using the counter electrode as the standard and the response current was measured.

Fig. 4 also shows the result of the measurement with the use of a conventional biosensor for comparison.

In the case of the conventional biosensor, 5 μL of a standard solution containing L-phenylalanine was added and, after 60 seconds, a potential was applied at -220 mV vs. Ag/AgCl by using the counter electrode as the standard and the response current was measured.

When a sample is added to the reaction reagent, the substrate in the sample undergoes a specific enzyme reaction under the action of the dehydrogenase and the coenzyme contained in the reaction reagent to thereby form the reduced coenzyme. Then a redox reaction quickly proceeds between this reduced coenzyme and the electron mediator and the tetrazolium salt and a chemically stable formazan is formed as the final product. Subsequently, a potential is applied to the electrode system and thus the formazan is electrochemically changed. Then the response current thus arising is detected. Since this response current depends on the substrate concentration, the substrate can be quantified thereby. Fig. 5 shows a reaction model view of the present invention as described above. Fig. 6 shows the fundamental structural formulae of tetrazolium salts and formazans.

As a result, a response of a very good linearity was achieved in an L-phenylalanine concentration range of from 0 to 1 mM. A very large response current showing a current density of about 120 $\mu A/cm^2$ per mM of L-phenylalanine was obtained.

Although use was made of a biosensor involving a two-

electrode system having a working electrode and a counter electrode in the above examples, quantification with a higher accuracy can be also made by using a three-electrode system with a reference electrode.

CLAIMS

1. A method of quantifying a substrate in a sample which comprises performing an enzyme reaction and a redox reaction between a reaction reagent comprising at least a dehydrogenase, a coenzyme, an electron mediator and a tetrazolium salt and the sample, and detecting a formazan formed as the final reaction product by using an electrode system made of electrically conductive materials.

2. The method as claimed in Claim 1 wherein said substrate is alanine, an alcohol, an aldehyde, isocitric acid, uridine-5'-diphospho-glucose, galactose, formic acid, glycerylaldehyde-3-phosphate, glycerol, glycerol-3-phosphate, glucose, glucose-6-phosphate, glutamic acid, cholesterol, sarcosine, sorbitol, carbonic acid, lactic acid, 3-hydroxybutyric acid, pyruvic acid, phenylalanine, fructose, 6-phosphogluconic acid, formaldehyde, mannitol, malic acid or leucine.

3. The method as claimed in Claim 1 wherein said formazan is electrochemically changed by applying a certain potential to said electrode system and the thus arising response current is detected.

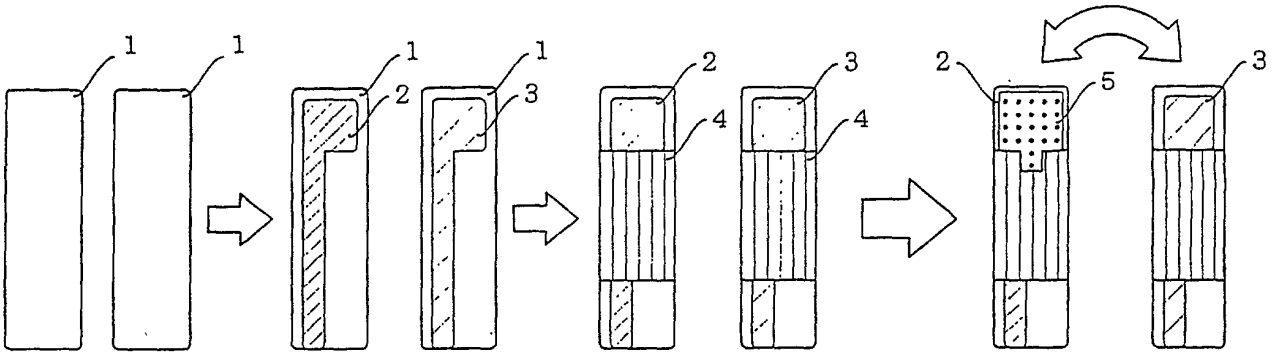
4. A biosensor for detecting said formazan by using the method as claimed in Claim 1 wherein said reaction reagent and electrode system consisting of at least a working electrode and a counter electrode made of electrically conductive materials are integrated.

5. The biosensor as claimed in Claim 4 wherein said formazan is electrochemically changed by applying a certain

potential to said electrode system and the thus arising response current is detected.

ABSTRACT

A method of quantifying a substrate, by which the substrate contained in various samples can be conveniently and quickly quantified without resort to any troublesome pretreatments, and a biosensor. More particularly speaking, a method of quantifying a substrate in a sample by using an electrode system made of electrically conductive materials and a reaction reagent comprising at least a dehydrogenase, a coenzyme, an electron mediator and a tetrazolium salt, which comprises performing an enzyme reaction and a redox reaction between the reaction reagent and the substrate in the sample, and detecting a formazan formed as the final reaction product by using the electrode system and a biosensor with the use of the same are provided.



1: Insulating support

2: Working electrode

3: Counter electrode

4: Insulating layer

5: Absorbent carrier

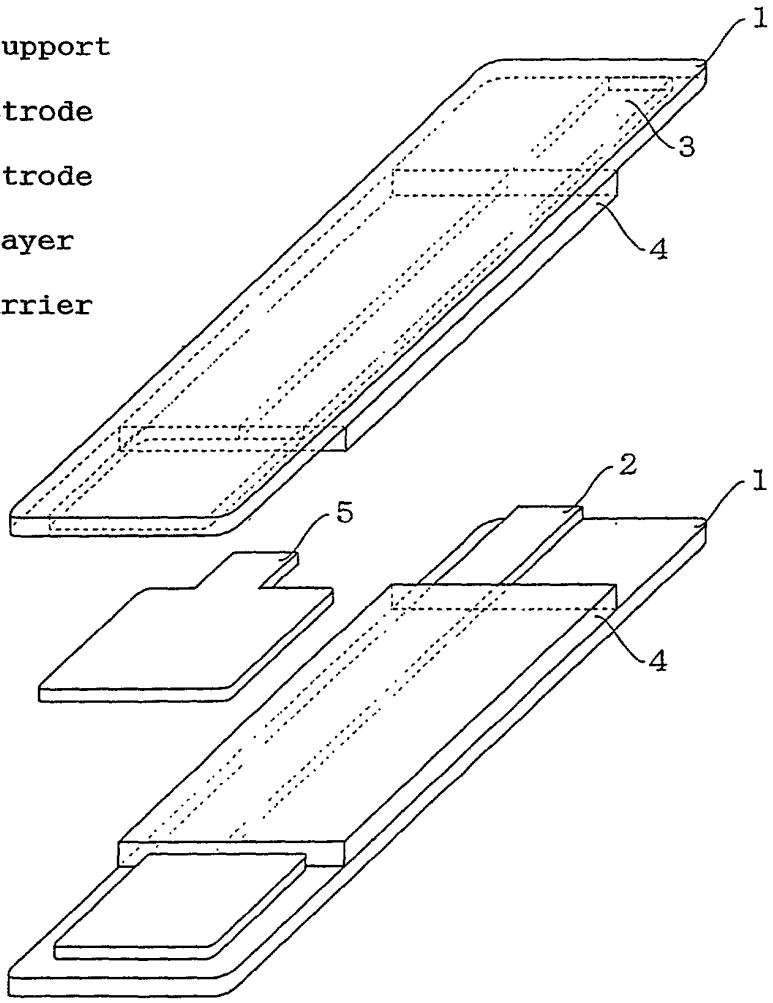


Fig. 1

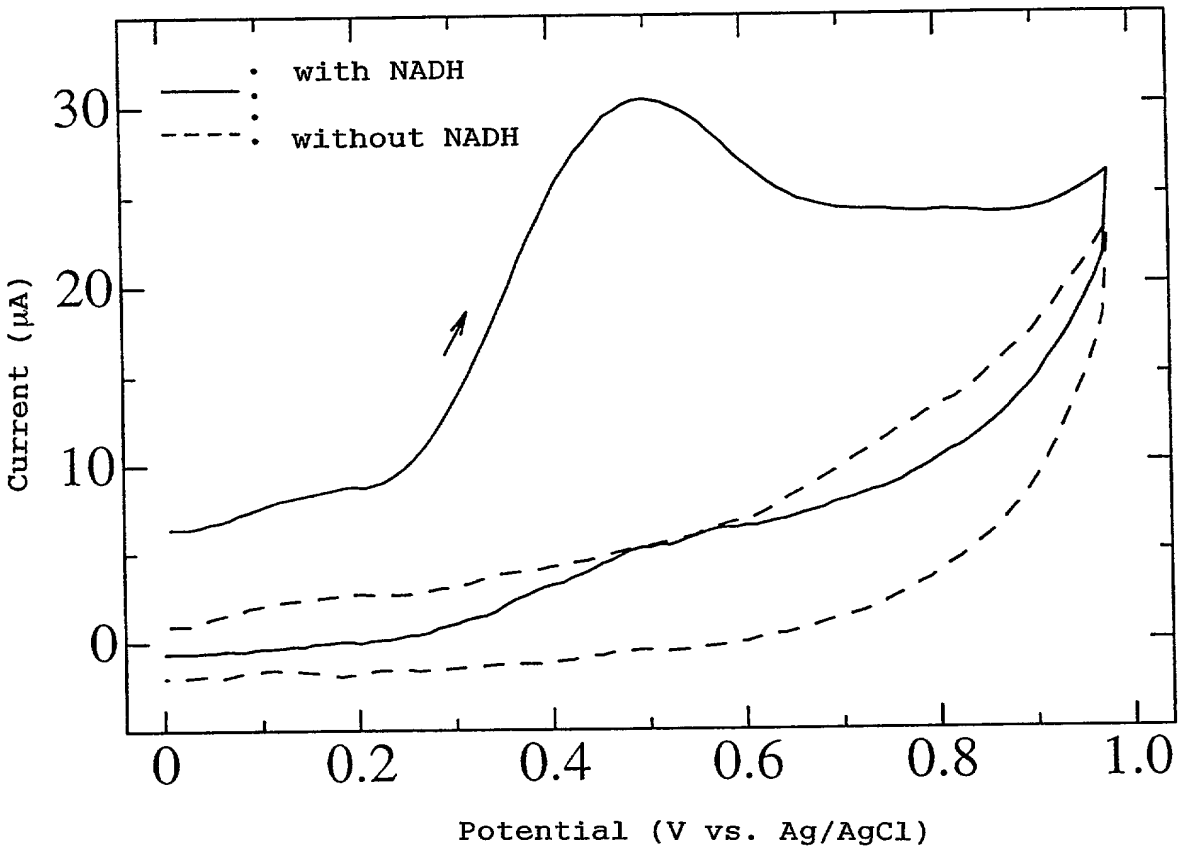

with NADH
without NADH
Current (μA)
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
Potential (V vs. Ag/AgCl)

Fig. 2

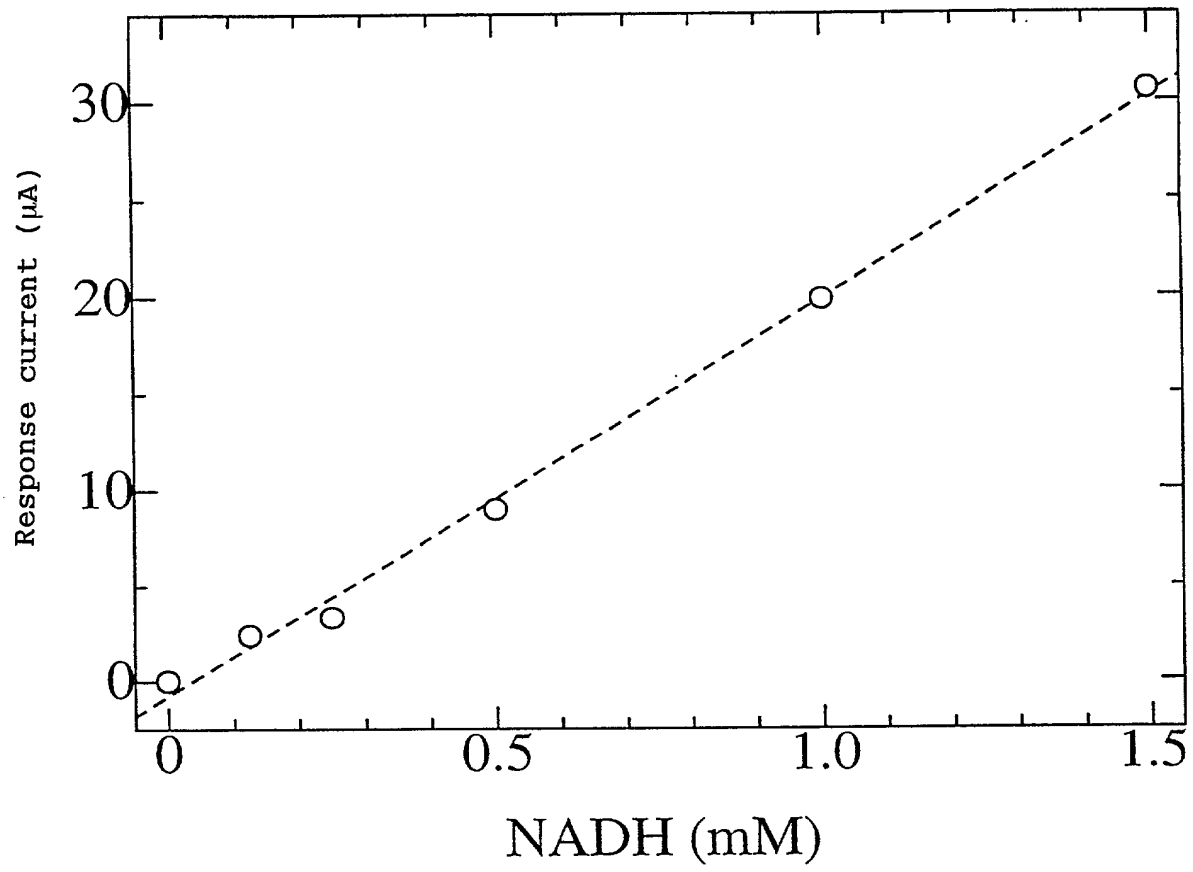


Fig. 3

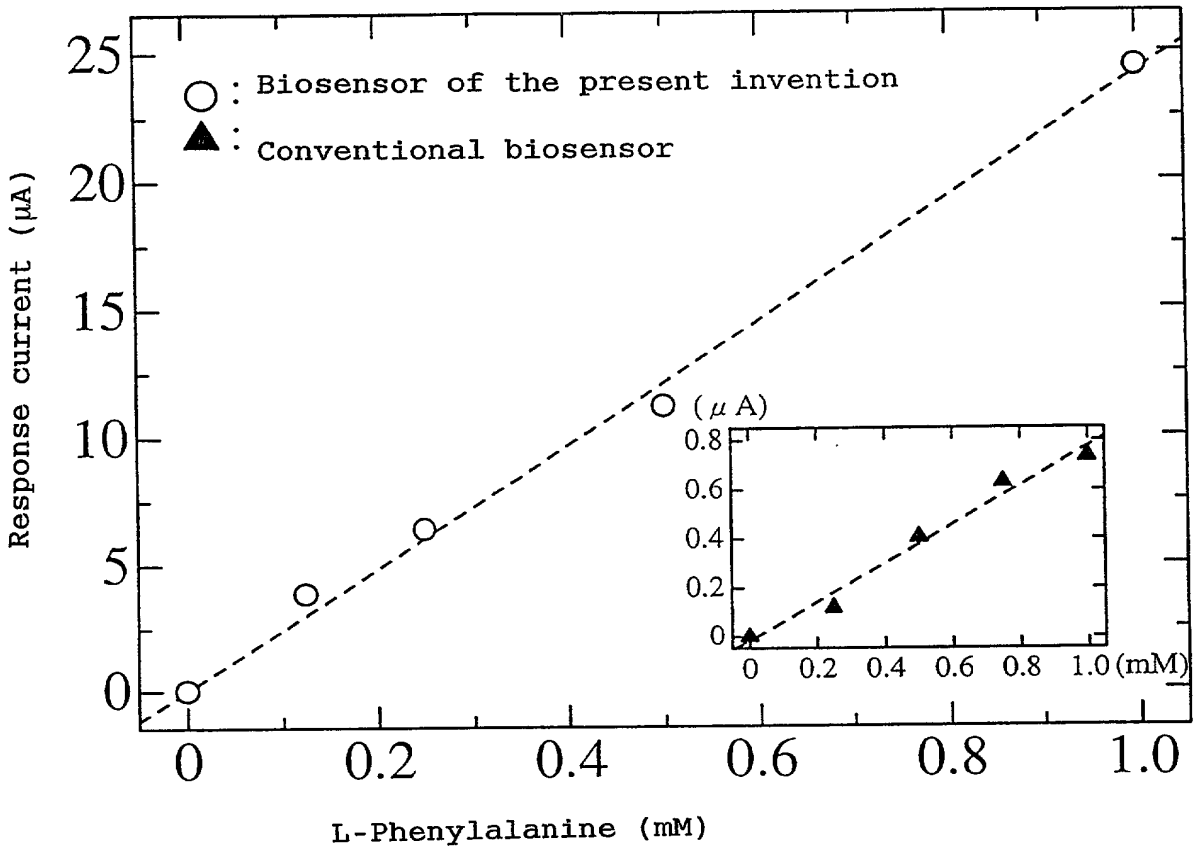

Response current (μA)
25
20
15
10
5
0
○ : Biosensor of the present invention
▲ : Conventional biosensor
0
0.2
0.4
0.6
0.8
1.0
L-Phenylalanine (mM)
(μA)
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
(mM)

Fig. 4

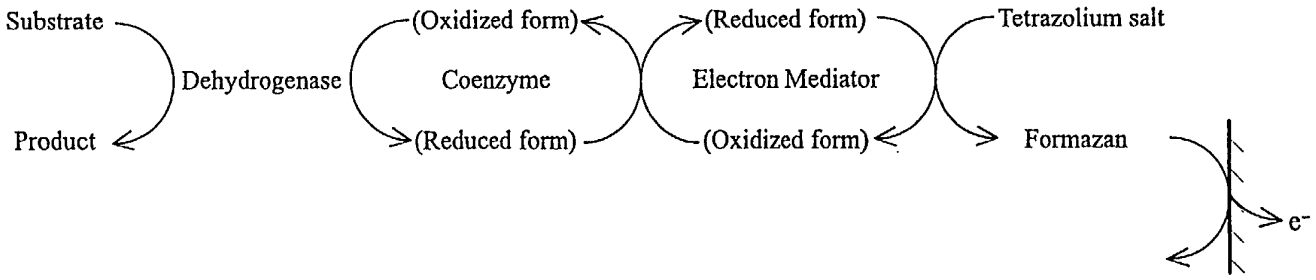
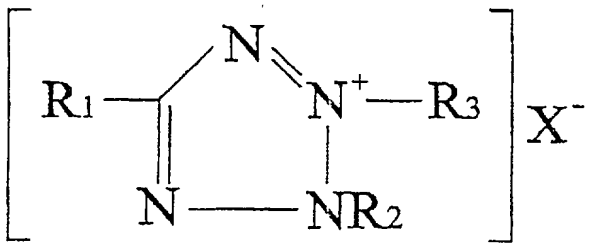
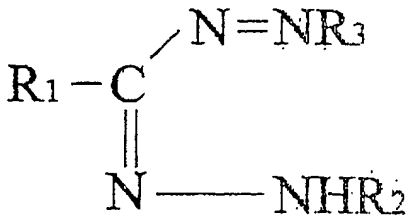


Fig. 5



Tetrazolium salt



Formazan

R_1, R_2, R_3 : alkyl group
X^- : halogen

Fig. 6

Case No. _____ Nixon & Vanderhye P.C. (12/97)

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:
METHOD OF QUANTIFYING SUBSTRATE AND BIOSENSOR

the specification of which (**check** applicable **box(s)**):
☐ is attached hereto
☐ was filed on _____ as U.S. Application Serial No. _____
☒ was filed as PCT International application No. PCT/JP99/01392 on March 19, 1999
and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:
Priority Foreign Application(s):

Application Number	**Country**	**Day/Month/Year Filed**

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

Application Number	**Date/Month/Year Filed**

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose material information as defined in 37 C.F.R. 1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

Prior U.S./PCT Application(s): Application Serial No.	**Day/Month/Year Filed**	**Status: patented pending, abandoned**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And I hereby appoint **NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8th Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed)**, and the following attorneys thereof (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Arthur R. Crawford, 25327; Larry S. Nixon, 25640; Robert A. Vanderhye, 27076; James T. Hosmer, 30184; Robert W. Faris, 31352; Richard G. Besha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffry H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam, Jr. 29366; Thomas E. Byrne, 32205; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; William J. Griffin, 31260; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334.

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FOR ADDITIONAL INVENTORS, check box ☐ and attach sheet with same information and signature and date for each.